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A two-photon probe for Al^{3+} in aqueous solution and its application in bioimaging

Haihong Wang, Bei Wang, Zhaohua Shi, Xiaoliang Tang, Wei Dou, Qingxin Han, Yange Zhang, Weisheng Liu *

Key Laboratory of Nonferrous Metals Chemistry and Resources Utilization of Gansu Province and State Key Laboratory of Applied Organic Chemistry, College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, PR China

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ABSTRACT

A salicylimine probe **L** with a simple structure has been researched more in-depth on fluorescence sensor properties based on two-photon (TP) absorption. **L** displays excellent selective turn-on fluorescence response for Al^{3+} in hexamethylenetetramine-buffered (HMTA) aqueous solution (0.3 M, pH=5.8) under one-photon (OP) excitation. With the help of OP fluorescence, TP fluorescence titration, UV-spectra titration and Job's plot, the stoichiometric ratio of **L** with Al^{3+} was determined to be 1:1. The coordination sites and the coordination mechanism of **L** with Al^{3+} were analyzed in detail through ^1H NMR data. Not only with a detection limit of 5.2×10^{-9} M *in vitro*, but also the probe has been successfully used in the live cells and tissues for the imaging of Al^{3+} with TP fluorescence microscopy due to the enlarged TP cross section, providing a novel testing method for measuring Al^{3+} in solution or cell tissue with low autofluorescence and cytotoxicity.

1. Introduction

Aluminum is the most abundant metallic element in earth's crust and its compounds are widely used in chemical industry production, from food additives, medicines, cookware, to production of light alloys, *etc*., which are closely associated with our daily life. It is universally known that Al is not the element people need and its accumulation can bring toxicity to the living body. When it comes to acid rain Al^{3+} which comes from the soil with poor buffer ability enters into the rivers and lakes, increasing the concentration of Al^{3+} in natural water and giving rise to the death of plants (Emmanuel and Ryan, 1995; Ren and Tian, 2007) and fishes (Alstad et al., 2005). In the human body Al^{3+} can be a competitive inhibitor of several essential elements, such as Mg^{2+}, Ca^{2+} and Fe^{3+} due to their similar characteristics (Das et al., 2012; Banerjee et al., 2012; Williams, 2002). An excess of Al^{3+} may have a bad effect on the central nervous system, further leading to dementia, myopathy, and Alzheimer's disease (Perl et al., 1982; Perl and Brody, 1980). In order to prevent the absorbtion of Al^{3+} and study in depth the key role of the metal ion in our body, it is of great importance to detect Al^{3+} *in vivo* and *in vitro* with different measuring methods.

In recent years, organic fluorescent probes were widely applied to detect biologically important ions for their generally non-destructive character, high sensitivity, instantaneous response, and the wide range of indicator dyes available (Amendola et al., 2006; Wang et al., 2010). Though many reports on the detection of Al^{3+} were published (Das et al., 2013), most of them were always problematic due to the lack of spectroscopic characteristics and poor coordination ability compared to transition metals (Soroka et al., 1987). Because Al^{3+} can be hydrolyzed easily to generate $Al(OH)_3$ precipitation most of the probes cannot be applied in water let alone in buffered solution which is beneficial to practical application, such as biological imaging (Zhao et al., 2006; Li et al., 2014).

So far, there have been some Al^{3+} fluorescent probes being applied to cell imaging, but all of them were measured with OP microscopy (Wang et al., 2010; Kim et al., 2012; Zhi et al., 2013) which required a rather short excitation wavelength and would produced background fluorescence (Guo et al., 2013; Sun et al., 2012) as well as photo-damage. In order to overcome the above limitations, TP microscopy, as a kind of new technology, has been used in this area recently. Multiple-photon excitation technology is the progress where two or more photons emitted by a long-wave light source can excite a short-wave one (Biswas et al., 2011). In contrast to OP microscopy, TP microscopy offers several distinct advantages: (1) small scattering effects can make light penetrate

* Corresponding author. Fax: +86 931 8912582.
E-mail address: liuws@lzu.edu.cn (W. Liu).

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samples deeper and more easily; (2) reduced photo-damage and photobleaching can make TP microscopy more suitable for viable cell observation (Biswas et al., 2011). Previously, scientists have spared no effort in applying TP microscopy to the field of TP fluorescence imaging (Taki et al., 2004; Dong et al., 2013; Zheng et al., 2014) and developed lots of new TP fluorescence probes with large TP absorption cross sections for the use in biological imaging (Li et al., 2011; Wang et al., 2012; Stewart et al., 2008; Cao et al., 2007; Zhu et al., 2010; Yong et al., 2009; Padilha et al., 2011; Chattopadhyaya et al., 2011; Shao et al., 2011; Poronik et al., 2013; Zhang et al., 2009; Nguyen et al., 2010; Chen et al., 2009; Feng et al., 2010). Cho and Kim et al. developed a series of TP fluorescence probes for detecting Na^+ (Kim et al., 2010b, 2010a), Ca^{2+} (Kim et al., 2010a, 2007b), Mg^{2+} (Kim et al., 2007a), Zn^{2+} (Kim et al., 2008), H^+ (Park et al., 2012; Lee et al., 2013), thiol (Lee et al., 2010), NO (Dong et al., 2013), *etc*., and applied them not only in living cells but also in tissues. In addition to this several other ions have also been studied, such as Cd^{2+} (Liu et al., 2012; Li et al., 2012), Hg^{2+} (Rao et al., 2012), Cu^{2+} (Fu et al., 2013), F^- (Zhang et al., 2011), *etc*. Though there were several studies on the TP absorption properties of aluminum complexes, they were mainly focused on theoretical study (Liu et al., 2004a; Zhang et al., 2007; Liu et al., 2004b; Yang et al., 2011). Calixarene-based TP fluorescent sensors have been synthesized by Cho et al. to sense metal ions Pb^{2+} and Al^{3+} but they cannot distinguish the two ions in CH_3CN (Kim et al., 2006), which is the most basic requirement probes should meet. Even so, to the best of our knowledge, there have not been any reports on TP fluorescence microscopy imaging of Al^{3+}.

Considering appealing applications of Schiff base derivatives in optical sensing, antitumor ability (da Silveira et al., 2008), antioxidative effects (Li and Yang, 2009), attractive electronic and photophysical property (Kasselouri et al., 1993) we researched more in-depth fluorescence sensor properties of a simple Schiff bases ligand N′-(2-hydroxybenzylidene)benzohydrazide (**L**), which has a large TP absorption cross section when adding Al^{3+}. **L** displays excellent selectivity for Al^{3+} in aqueous solution and it can also be used for living cell and tissue imaging.

2. Materials and methods

2.1. Materials and instrumentation

All reagents and solvents employed for synthesis were commercially available and used as received. Deionized water was used as solvent. All of the solvents used were of analytical reagent grade. The solutions of metal ions were prepared from either their nitrate or their chloride salts. HMTA buffered aqueous solution (0.3 M, pH=5.8) was prepared in double-distilled water. ^1H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Bruker DRX400 spectrometer in d_6-DMSO and d_4-CH_3OH with TMS as internal standard. Mass spectra was measured on a Bruker Esquire 6000 mass spectrometer by means of the electronic spray ionization (ESI) technique. Absorption spectra were recorded using a Varian Cary 100 spectrophotometer. Fluorescence measurements were made on a Hitachi F-7000 fluorescence spectrophotometer equipped with a xenon lamp as the excitation source. The path length was 1 cm with a cell volume of 3.0 mL. Excitation and emission slits of 2.5 nm were used for the measurements of fluorescence. Elemental analyzes were conducted using an Elemental Vario (EL) instrument. Melting point was determined on an X-6 melting point apparatus without calibration (Beijing Fuka Keyi Science and Technology Co., Ltd.). Fluorescent quantum yields were determined by an absolute method using an integrating sphere on FLS920 of Edinburgh Instrument. All pH measurements were made with a pH-10 C digital pH meter. All the measurements have been done at room temperature unless otherwise stated.

2.2. Optical detection of Al^{3+}

The probe **L** (10.0 μM) was mixed with different concentrations of metal ions, 10.0 μM $Al(NO_3)_3$ and $Ga(NO_3)_3$, 50.0 μM NaCl, KCl, $AgNO_3$, $Cr(ClO_4)_3$, $Co(ClO_4)_2$, $Ni(NO_3)_2$, $FeCl_3$, $Cu(NO_3)_2$, $CaCl_2$, $Mn(ClO_4)_2$, $ZnCl_2$, $LiClO_4$, $Cd(NO_3)_2$, $MgCl_2$ and $Hg(ClO_4)_2$ in HMTA buffered aqueous solution (0.3 M, pH=5.8). After equilibrium at ambient temperature for 25 min, absorption and fluorescence spectra of the mixtures were measured. Fluorescence spectra were measured at an excitation wavelength of 366 nm, and emission spectra were collected from 390 to 600 nm.

2.3. Measurement of TP cross section (δ)

TP excitation fluorescence spectra were measured using a Steady-state and Lifetime Fluorescence Spectrometer (FLS920, Edinburgh Instruments). TP absorption cross sections (δ) at different excitation wavelengths were determined by comparing their TP excitation fluorescence with that of fluorescein (Albota et al., 1998) in aqueous solution, according to the following equation:

$$\delta = \delta_{ref}\Phi_{ref}/\Phi c_{ref}/cn_{ref}/nF/F_{ref} \quad (1)$$

In Eq. (1), the subscript *ref* stands for the reference molecule. δ is the TP absorption cross section value, c is the concentration of solution, n is the refractive index of the solution, F is the TP excitation fluorescence integral intensities of the solution emitted at the exciting wavelength, and Φ is the fluorescence quantum yield. The δ_{ref} value of reference was taken from the literature (Li et al., 2012).

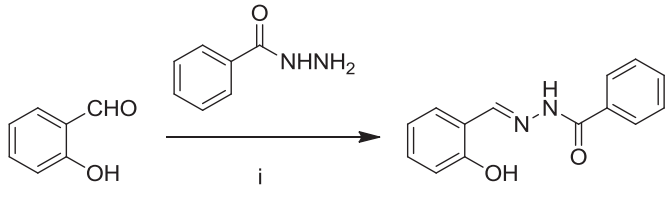
2.4. TP fluorescence imaging

TP fluorescence images of **L** labeled cells and tissues were obtained by exciting the probes with a modelocked titanium–sapphire laser source (Mai Tai DeepSee, 80 MHz, 90 fs) set at wavelength 730 nm with AN Olympus FV1000 laser confocal microscope IX81 with 60× objective, numerical aperture (NA)=0.4. The images signals in 420–470 nm range were collected by internal PMTs in 12 bit unsigned 1024×1024 pixels at 40 Hz scan speed.

3. Result and discussion

3.1. Synthesis of **L**

L was facilely synthesized from the reaction of benzohydrazide with salicylaldehyde (Scheme 1). The molecular structure and its purity were confirmed by NMR and ESI-MS.



L

Scheme 1. Synthesis of the fluorescent indicator **L**. Reagents and conditions: (i) ethanol, reflux, 8 h, 82%.